

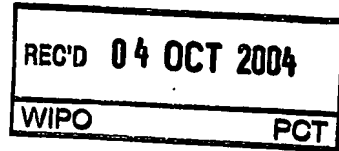
05.07.07



Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets



Bescheinigung

Certificate

Attestation

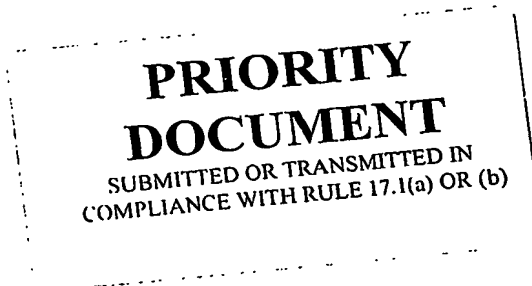
Die angehefteten Unterla-  
gen stimmen mit der  
ursprünglich eingereichten  
Fassung der auf dem näch-  
sten Blatt bezeichneten  
europäischen Patentanmel-  
dung überein.

The attached documents  
are exact copies of the  
European patent application  
described on the following  
page, as originally filed.

Les documents fixés à  
cette attestation sont  
conformes à la version  
initialement déposée de  
la demande de brevet  
européen spécifiée à la  
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03254472.8



Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

R C van Dijk

BEST AVAILABLE COPY



Europäisches  
Patentamt

European  
Patent Office

Office européen  
des brevets

PCT/EP200 4/ 0 0 7 2 9 7

05.07.04

Anmeldung Nr:  
Application no.: 03254472.8  
Demande no:

Anmeldetag:  
Date of filing: 17.07.03  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

UNILEVER PLC  
Unilever House,  
Blackfriars  
London EC4P 4BQ  
GRANDE BRETAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se referer à la description.)

Method for testing ghrelin release

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

C12N5/00

Am Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL  
PT RO SE SI SK TR LI

## METHOD FOR TESTING GHRELIN RELEASE

### Field of the invention

The present invention relates to the use of a cell line,  
5 preferably RF-1 or RF-48, derived from a human gastric  
adenocarcinoma of human caecum, to measure regulation of  
expression and/or secretion of a peptide called ghrelin.  
Ghrelin, the length of which is 28 amino acids, is the ligand  
for the growth hormone receptor. Ghrelin is known to exist in  
10 at least two forms:

1) n-octanoyl ghrelin in which the third serine residue is n-  
octanoylated and 2) des-n-octanoyl ghrelin in which the n-  
octanoyl group is removed. These two forms of ghrelin have  
different physiological functions.

15

### Background of the invention

Although ghrelin has only been identified in 1999, more than  
200 papers and articles on the substance have already been  
published, indicating the importance of this peptide hormone.  
20 Ghrelin is the first identified peripheral hormone signalling  
appetite. People who were given ghrelin increased their  
appetite resulting in up to one third more food intake than  
control subjects. This earned ghrelin the name, the "hunger  
hormone".

25

In addition to stimulating food intake, ghrelin levels drop  
once an individual starts eating. Consequently, it is believed  
that ghrelin acts as a trigger to start food intake.

Furthermore, data have been reported showing that ghrelin  
30 levels do not fall after eating in obese individuals which  
suggests that this trigger is not reset in such individuals.

2

It has also been suggested in the past that ghrelin could affect our body's internal clock, thus making us feel hungry at certain times during the day.

5 Many people all over the world suffer from disturbances in their appetite and, as a consequence, their food intake. When energy intake and energy expenditure are in imbalance, body weight changes which can have severe physiological  
10 group of overweight or obese people. In most cases, their excessive body weight is a consequence of increased food intake in combination with decreased energy expenditure. On the other hand there is a group of people suffering from decreased appetite and low body weight. This can be due to illness such  
15 as cancer or anorexia, but is also observed in many elderly people.

Decreasing the level of ghrelin in an individual could inhibit hunger feelings and thereby help people, particularly  
20 overweight or obese people, to decrease their food intake and lose weight. In contrast, increasing the level of ghrelin could boost appetite and stimulate food intake in ill or old people, generally improving their health and wellbeing.

25 The properties and characteristics of ghrelin could pave the way to the formulation of a drug that either blocks or increases the level and/or activity of this hormone.

Modulation of ghrelin levels and/or activity could also be used  
30 as a basis for developing functional ingredients for food products that modulate feelings of satiety or appetite in humans or animals.

Discovery of such drugs or ingredients would be facilitated by an in vitro model system that mimics the production pathway of ghrelin.

5 Ghrelin is mainly produced in the stomach, but it also exists in parts of the brain that controls the appetite.

Ghrelin has furthermore been shown to occur in endocrine tumors of the human stomach, pancreas, intestine and lungs (G.Rindi et al (Histochem Cell Biol. 2002 June, 117(6), 521-5). Regulation  
10 of the expression, synthesis, and secretion of ghrelin is however still largely unknown.

It is an object of the present invention to find a screening method for assessing the effect of test compounds on the level  
15 and activity of ghrelin.

It is another object of the invention that this screening method is simple and reproducible.

It is a further object of the invention to provide a method for analysing the secretion of both forms of ghrelin, in particular  
20 for examining the endogenous molecules that regulate their synthesis and secretion.

It is a still further object of the invention to provide a method for analysing regulation of ghrelin gene expression.

It is also an object of the invention that this screening  
25 method can be used for estimating the effect on food intake of a variety of test compounds, which were found to suppress or stimulate the release of ghrelin in vitro.

We have now surprisingly found that these objects can be  
30 achieved by a cellular model for studying the level of ghrelin production. This model comprises a cell line derived from a human gastric adenocarcinoma, which can release ghrelin upon growth in a suitable medium.

Definition of the invention

Accordingly, in a first aspect the present invention provides a model to study the (regulation of) expression, synthesis and/or secretion of ghrelin, comprising in vitro culture of a cell line derived from a gastric adenocarcinoma, said cell line being capable of producing ghrelin, and said model also comprising a medium for growing said cell line. The model of the invention is particularly useful for finding compounds which can modulate appetite in humans. However, the model can also be used for screening compounds that can modulate appetite in animals, and that could be added to functional pet food or feed.

In a second aspect, the present invention provides a method for assessing the (regulation of) expression, synthesis and/or secretion of ghrelin, wherein a cell line derived from a gastric adenocarcinoma and capable of producing ghrelin when grown in a suitable medium, is grown in such medium. In the method of the invention, the cell line is preferably incubated with a test compound, and the effect of the test compound on the production of ghrelin is preferably measured.

In a third aspect, the present invention provides the use of a cell line derived from a gastric adenocarcinoma that is capable of producing ghrelin when grown in a suitable medium, for assessing the (regulation of) ghrelin expression, synthesis and/or release.

30 Detailed description of the invention.

We have found that the cell lines used in the present invention are suitable for measuring expression, synthesis and/or secretion of ghrelin. Furthermore, the model and method of the

invention appeared to be suitable for screening a large number of test compounds with regard to their effect on ghrelin expression and/or secretion. This effect can either be a stimulating effect or a blocking effect.

- 5 It has also been found that the method of the invention is suitable for screening simultaneously a number of test compounds that are expected to modulate (i.e. stimulate or block) ghrelin expression and/or secretion, at various concentrations. Furthermore, application of the method of the  
10 invention can provide an estimation of the effect of various test compounds on food intake in humans or animals thereby decreasing the complexity of in vivo feeding trials.

The method and model of the invention were found to be  
15 particularly suitable for use with a cell line derived from a human gastric adenocarcinoma. Preferably, the cell line is selected from RF-1 having ATCC number CRL-1864 and RF-48 having ATCC number CRL-1863.

- 20 Preferably, the medium for growing the cell line used in the present invention is Leibovitz's L15 containing 10% (vol/vol) foetal bovine serum and 2 mM L-glutamine, and the cell line is desirably grown in said medium at a temperature of 37° C and in the absence of CO<sub>2</sub>.

25

- The medium for growing the cell line is effectively changed at least every 4 days, preferably every 3-4 days, until cell confluence is achieved. Subsequently, the cells are suitably grown and plated in 24-well cultures plates whereby each well  
30 may contain  $1 \times 10^4$  to  $5 \times 10^6$  cells. The cells could also be grown in cultures plates containing different numbers of wells, such as 48 wells or 96 wells.

Preferably, these plates are stored under the same incubation conditions as those used for growing the cell line.

In the screening method of the invention, the cells are  
5 subsequently exposed to a variety of test compounds, whereby  
ghrelin release may effectively be measured using an  
immunoassay kit. Preferably, for each treatment with a test  
compound three samples were prepared. In other words, the  
effect on ghrelin release by each type of test compound is  
10 desirably measured in triplicate. In order to be able to make  
reliable comparisons it is also desirable to prepare a control  
sample that does not contain any test compound, in triplicate.

In the screening method of the invention the effect of the test  
15 compounds on ghrelin release may effectively be measured as  
follows. The cultures plates are stored as described above and,  
after 0.5 -3 hours incubation, a sample is taken from each well  
to measure ghrelin release. Each sample is centrifuged at 1000-  
5000 rpm during 1-10 minutes to remove the cells from the  
20 sample. The supernatant containing the medium, the test  
compound and, additionally, any ghrelin secreted is then  
transferred to a separate tube. The thus-formed supernatant  
samples are suitably frozen, e.g. at -80 °C, for subsequent  
analysis of ghrelin secretion. The supernatant samples may also  
25 not be frozen and directly used in the measuring device.

Ghrelin release can be suitably measured using an immunoassay  
kit. This kit may be a commercial enzyme immunoassay -EIA- kit  
(from Phoenix Pharmaceutical, Belmont, California, USA).  
30 Alternatively, two types of radioactive immunoassay (RIA) kits  
(from Linco Research Inc., Missouri, USA) are available with  
which either the level of n-octanoyl ghrelin or the total level



of ghrelin released (i.e. both forms, see above) can be measured.

The cells according to the present invention can additionally  
5 be used in a screening method for assessing the effect of test  
compounds on ghrelin gene expression, preferably by means of  
quantitative RT-PCR (Reverse Transcription -Polymerase Chain  
Reaction). The cells can furthermore be used to assess  
endogenous molecules that regulate ghrelin expression,  
10 synthesis or secretion. Finally, the cells of the present  
invention can be used in a method to test the effect of  
compounds that may modulate ghrelin activity.

### **Examples**

15 The following compounds were tested with regard to their  
ability to stimulate ghrelin release:

- Tyr(SO<sub>3</sub>H]<sub>27</sub>)Cholecystokinin fragment 26-33 Amide (referred to as CCK); and
- Gastrin I human (referred to as Gastrin).

20

These test compounds were made as 100X stock solutions by  
dissolving them in pure ethanol, as a vehicle. The test  
compounds were then diluted 1/100 in the Leibovitz L-15 medium  
containing 0.5% foetal bovine serum (FBS).

25

Since the effect caused by these test compounds on ghrelin  
release by a RF-48 cell line was tested, RF-48 cells were grown  
during incubation at 37° C in Leibovitz's L15 medium with 2 mM  
L-glutamine and containing 10% (vol/vol) FBS in the absence of  
30 CO<sub>2</sub>.

After cell confluence was obtained, the cells were plated in 24-well cultures plates ( $1 \times 10^6$  cells/well). Several wells were subsequently exposed to one of the test compounds as prepared above. For each type and concentration of test compound tested, 5 a series of three tests was carried out.

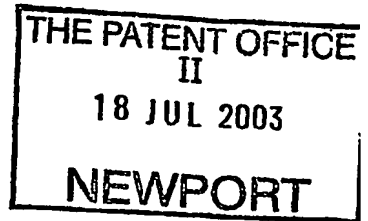
After 1 hour of incubation of the thus-filled wells containing both the cells and the test compound at the same conditions as those applied during growing of the RF-48 cells, a sample was 10 taken from each well to measure ghrelin release.

Each sample was centrifuged at 3000 rpm to remove the cells from the sample and the supernatant (containing the ghrelin formed as well as the medium and the test compound) was transferred to a separate tube. The ghrelin release was 15 measured using a commercial enzyme immunoassay kit (from Phoenix Pharmaceuticals, Belmont, California, USA).

The results are shown in Figure 1, showing the amount of ghrelin released from RF-48 cells as a function of the type and 20 concentration of the test compound tested.

In Figure 1, the concentration of the test compound is shown in micromols per litre ( $10^{-6}$  M) and the concentration of the ghrelin formed in the tested samples is shown in nanograms/ml. Figure 1 also provides the results of a control experiment, 25 showing the ghrelin release when no test compound is present in the sample containing the RF-48 cells. In addition, the results of a second control experiment are provided -indicated as "Control +veh" -, also showing ghrelin release when no test compound is present in the sample containing the RF-48 cells 30 but whereby 1% of pure ethanol has been added to said sample.

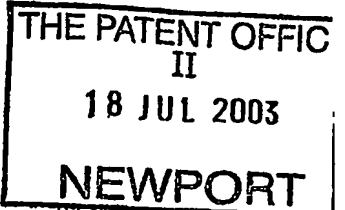
It can be clearly noticed that both CCK and Gastrin stimulate the release of ghrelin. It can also be seen that both control experiments show similar levels of ghrelin release, which clearly indicates that the ethanol vehicle does not stimulate  
5 ghrelin release.



**Claims**

1. A model for the study of the (regulation of) expression, synthesis and/or secretion of ghrelin, comprising in vitro culture of a cell line derived from a gastric adenocarcinoma, said cell line being capable of producing ghrelin, and said model also comprising a medium suitable for growing said cell line.
2. A model according to claim 1, wherein the cell line is selected from RF-1 having ATCC number CRL-1864 and RF-48 having ATCC number CRL-1863.
3. A method for assessing the (regulation of) expression, synthesis and/or secretion of ghrelin, wherein a cell line derived from a gastric adenocarcinoma and capable of producing ghrelin when grown in a suitable medium, is grown in such medium.
4. A method according to claim 3, wherein the cell line is selected from RF-1 having ATCC number CRL-1864 and RF-48 having ATCC number CRL-1863.
5. A method according to claim 3 or 4, wherein the medium is Leibovitz's L15 containing 10% foetal bovine serum and 2 mM L-glutamine, and wherein the cell line is grown at a temperature of 37° C in the absence of CO<sub>2</sub>.
6. A method according to any of claims 3-5, wherein the medium is changed at least every 4 days.

7. A method according to any of claims 3-6, wherein the cell line is plated and grown in a culture plate after achieving cell confluence, wherein the plate is stored under the same incubation conditions as those used for growing the cell line, and wherein ghrelin production is measured using an immunoassay kit.
8. Method according to claims 3-6, wherein the cell line is used to study ghrelin gene expression, preferably by means of quantitative RT-PCR.
9. A method according to any of claims 3-8, wherein the cell line is exposed to a variety of test compounds.
10. The use of a cell line derived from a gastric adenocarcinoma that is capable of producing ghrelin when grown in a suitable medium, for assessing ghrelin secretion in vitro.
11. The use according to claim 10, wherein the cell line is selected from RF-1 having ATCC number CRL-1864 and RF-48 having ATCC number CRL-1863.

**Abstract**

The present invention provides a model to study the (regulation of) expression, synthesis and/or secretion of ghrelin in humans. Ghrelin is a hormone that signals appetite and stimulates food intake.

The model of this invention comprises the culture of a cell line derived from a human gastric adenocarcinoma that is capable of producing ghrelin, said model also comprising a medium suitable for growing said cell line. The invention also provides a method for assessing ghrelin production, wherein a cell line derived from a human gastric adenocarcinoma can synthesize and secrete ghrelin when grown in a suitable medium. This method can be used as screening tool when measuring the effect of test compounds on the production of ghrelin. Such screening tool allows for testing a large number of compounds in order to develop drugs or food ingredients that help to control food intake by regulating appetite.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**